

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

PDGFRs expression in dogs affected by malignant oral melanomas: correlation with prognosis

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1530759> since 2017-05-15T12:02:18Z

Published version:

DOI:10.1111/vco.12190

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

PDGFRs expression in dogs affected by malignant oral melanomas: correlation with prognosis

S. Iussich, L. Maniscalco, A. Di Sciuva, B. Iotti, E. Morello, M. Martano, F. Gattino, P. Buracco and R. De Maria

Department of Veterinary Sciences, University of Turin, Turin, Italy

Abstract

Canine malignant melanoma (CMM) is the most common canine oral tumour, and up to 70–75% of dogs in stage II–III die within 1 year after surgery. The purpose of this study was to evaluate the expression of platelet-derived growth factors receptors (PDGFR)- α and - β in stage II and III CMMs and to correlate it with prognosis. PDGFRs expression was evaluated by immunohistochemistry on 48 cases of formalin-fixed CMM samples and correlated with clinical–pathological findings and outcome after surgery. PDGFRs co-expression was observed in 37.5% of cases. Positivity for PDGFR- α and - β receptor was present in 54.2 and 47.9% of cases, respectively. Ki67 values >19.5% were ascertained in 66.7% of cases. Statistical analysis showed that PDGFRs co-expression and Ki67 values >19.5% were both associated with worse prognosis. PDGFRs expression suggests a role in the pathogenesis and progression of CMM, and α and β co-expression appears to be associated to worse prognosis.

Keywords

canine malignant melanoma, PDGF receptors, prognostic factor, targeted therapy

Introduction

Malignant melanoma (MM) represents the most frequent oral neoplasm occurring in dogs.^{1–3} Oral canine MM (CMM) has an aggressive behaviour, grows rapidly, is locally invasive, frequently metastasizes to regional lymph nodes (RLNs) and distant sites, and it may recur following surgical resection. Nuclear atypia, mitotic index and Ki67 index are the prognostic factors that are known to be most significant.⁴ The molecular alterations involved in CMM arising from mucosal or digital sites have not been yet fully identified. Recently, Gillard *et al.*⁵ used cDNA sequencing data from 95 dogs to detect somatic mutations in NRAS and PTEN genes at human hotspot sites, while no mutations were found in the analysis of BRAF Exon 15,⁶ as frequently occurs in human melanomas.^{7,8}

Platelet-derived growth factors receptors (PDGFR- α and PDGFR- β) are tyrosine kinases receptors that can activate many of the major signal transduction pathways, including

phosphatidylinositol 3-kinase (PI3K), Ras, mitogen-activated protein kinase (MAPK) and phospholipase C γ pathways.⁷

They are involved in physiological and pathological diseases mainly by paracrine mechanisms. In the physiological processes of adults, they stimulate fibroblast and endothelial cell proliferation and are involved in tissue regeneration and fibrotic processes; during embryogenesis they are responsible for tissue differentiation.^{8,9} In human cancers PDGFRs can be activated by various genetic alterations,^{10,11} and tumours of mesenchymal, glial and haematopoietic origin may show PDGFRs dysfunctions.¹² The most frequent alterations are over-expression, constitutive activation of the tyrosine kinase domain as well as post-transcriptional regulation by specific RNA sequences such as miRNA 34.¹³ The dysfunction of tyrosine kinases occurs frequently in human cancers, and more studies indicate that a similar pattern of dysfunction may also be observed in canine and feline

Correspondence address:

L. Maniscalco
Department of Veterinary Sciences
University of Turin
Largo P. Braccini 2,
Grugliasco
Turin, Italy
e-mail:
lorella.maniscalco@unito.it

cancers.^{14,15} In domestic animals PDGFRs have been studied in canine osteosarcoma, lymphoma, apocrine gland carcinoma, glioma and hemangiosarcoma but their expression has not been found to be correlated to prognosis.^{16–20}

The aim of this research was to evaluate the expression of PDGFR- α and - β in CMM, in order to identify their role in the tumour pathogenesis and their possible correlation with prognosis.

Materials and methods

Sample collection and clinical follow-up

The tissue samples examined were from spontaneous oral CMMs, treated between 1998 and February 2014 at the Department of Veterinary Sciences of the University of Turin. In all cases, the initial data collected included patient history, physical examination, blood cell count, serum biochemistry and urinalysis. Fine needle aspiration of palpable RLNs, even if not enlarged (as size has not been considered sufficiently predictive)²¹ and/or biopsy of the primary lesion were used for preoperative tumour diagnosis. A definitive and more objective staging was achieved in all cases via the surgical removal of all palpable RLNs at the time of primary tumour resection and their full histological evaluation. Full tumour staging included a skull and three-view chest radiographs and abdominal ultrasound examination; alternatively, a total body CT-scan was performed. Dogs without concurrent life-threatening diseases but with histologically confirmed stage II (2–4 cm diameter, negative RLN) or III (>4 cm diameter and negative RLN or any tumour size with regional-positive surgically resected RLN)²² oral CMM were included in the study. All the animals were followed until the recurrence of the neoplasm, death or for a minimum of 12 months after surgery. Together with the regional lymphadenectomy, a primary tumour *en bloc* resection was performed, with the inclusion, when feasible, of at least 2 cm of macroscopically normal tissue around the tumour.

Histopathology

Formalin-fixed and paraffin-embedded sections were subjected to haematoxylin/eosin staining

and histopathological examination was performed by two independent pathologists (S. I.–L. M.), recording mitotic index, degree of nuclear atypia and amount of pigmentation.^{4,23,24} In order to determine the melanocytic origin of the tumours, each sample was tested for PNL-2 expression.^{2,4}

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was carried out on 4 μ m sections of formalin-fixed, paraffin-embedded samples. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections underwent high-temperature antigen unmasking by incubation at 98 °C with citric acid buffer (pH 6.0). Samples were immunohistochemically tested for Ki67, PNL-2, PDGFR- α and β expression. The details of primary antibodies employed and the dilutions used are summarized in Table 1. Antibodies were detected using the avidin–biotin–peroxidase complex technique with the Vectastain Elite ABC Kit (Vector Laboratories). The following external positive controls were used: canine skin for PDGFR- α and canine prostatic carcinoma for PDGFR- β . As an internal positive control, endothelial cells of the normal blood vessels were used. For negative controls, the sections were incubated in the absence of the primary antibodies. Immunolabelled slides were randomized and masked for blinded examination, which was performed independently by two observers (L. M. and S. I.); in case of disagreement, a consensus was reached using a multi-head microscope. Cytoplasmic positivity was evaluated in both tumour and stromal cells, located separately using the scoring system adopted by Donnem *et al.* (2008).²⁵ Immunostaining at the stromal level was

Table 1. Source and conditions of the antibodies employed

Antibody	Type	Source	IHC
PDGFR- α	Rabbit polyclonal	Santa Cruz Biotechnology	1:200
PDGFR- β	Rabbit polyclonal	Santa Cruz Biotechnology	1:200
Ki67	Rabbit polyclonal	Dako	1:25
PNL-2	Rabbit polyclonal	Santa Cruz Biotechnology	1:25

considered as cytoplasmic labelling in the fibro-connective tissue that forms bundles within and surrounding the tumour.

Ki67 was evaluated considering the cut-off of 19.5 positive cells in five $\times 400$ fields.²⁴

Statistical analysis

IHC results and clinical-pathological findings were grouped into contingency tables and analysed using Pearson's Chi-squared test with Yates' continuity correction. Survival curves were computed using the Kaplan-Meier method and tests for differences in survival, considering all known prognostic factors for CMM, were performed using the log-rank test. Co-expression and presence of Ki67 values greater than 19.5% were evaluated in interaction using a Cox proportional hazard regression model. Overall survival (OS) was considered as the number of days between surgery and death, while the disease-free interval (DFI) as the number of days between surgery and tumour recurrence and/or evidence of metastasis. Cases that were still alive or that did not present tumour recurrence or metastasis at the end of the monitoring period (minimum 12 months), or that died for unrelated causes, were considered as censored. Data were analysed with R (R Core Team (2014). R: R Foundation for Statistical Computing, Vienna, Austria); *P* values less than 0.05 were considered statistically significant.

Results

Epidemiologic and clinical data

The data presented here come from 48 cases of oral CMMs. The mean age of dogs was 11.4 years (range: 5–14 years); 64.6% of the dogs (31/48) were males and 35.4% (17/48) females. Fifty percent of dogs were mixed breed, while 50% were pure breeds. The latter included: five dachshunds (10.4%), four Cockers (8.3%), three German Shepherds (6.3%), three Golden Retrievers (6.3%) and one each of (2.1%) Syberian Husky, Beagle, Dogue de Bordeaux, Greyhound, Yorkshire terrier, Schnauzer, Miniature Schnauzer, West Highland White Terrier and Labrador Retriever.

A total of 20 dogs had a stage II oral CMM and 28 a stage III oral CMM. All dogs underwent

surgical excision. Histology revealed incomplete excision margins in 13 dogs (27.1%). The median DFI recorded was 196 days (range 30–992 days) and the median OS was 258 days (range 70–992 days). Five censored cases were included in this study: two died for unrelated causes (euthanasia for orthopedical problems in one dog and *ab ingestis* pneumonia caused by idiopathic megaesophagus in the second dog) while three dogs were still alive at the end of the monitoring period.

Histopathology

Histopathology revealed that 30/48 cases (62.5%) of CMMs were characterized by the presence of melanin, while 18/48 (37.5%) were amelanotic. Regarding the histotype, 14 CMM were spindle-shaped (29.2%), 12 epithelioid (25%) and 22 mixed (45.8%).

IHC analysis

All samples analysed showed positivity to PNL-2 antigen, confirming the diagnosis of melanoma.⁴ Positivity for Ki67 was <19.5% in 16 cases (33.3%) and >19.5% in 32 cases (66.7%).

Immunolabelling for both PDGFR- α and - β receptors was observed at cytoplasmic level and diffusely within the tumour (Figs. 1 and 2). PDGFR- α and β expression was observed in 26/48 (54.2%) cases and 23/48 (47.9%), respectively. Among the 48 cases analysed, 15 (31.2%) were negative for both the PDGF receptors, 18 samples (37.5%) were positive to both PDGFR- α and - β , 8 (16.7%) were positive to PDGFR- α and negative to PDGFR- β , while seven (14.6%) were positive to PDGFR- β only. Regarding the positivity in the stromal cells compartment, PDGFR- α was present in 13/48 cases (27.1%), PDGFR- β in 8/48 (16.7%); of those, 5/48 (10.4%) were positive for both receptors.

Statistical analysis

Dogs with oral CMM expressing both PDGFR- α and - β had a statistically significant lower DFI (median 159 days versus 239 days, *P* < 0.05) and a lower OS (median 183 days vs. 335 days, *P* < 0.05) compared with dogs with CMM not co-expressing these receptors (Fig. 3). Also, a high Ki67 index

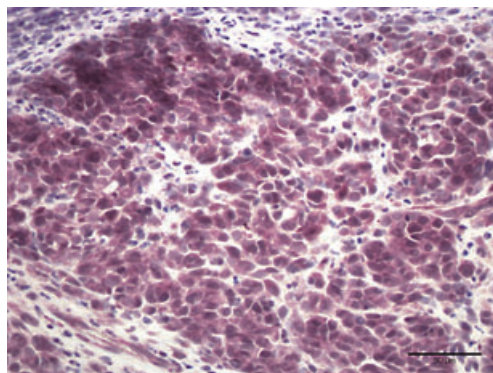


Figure 1. Malignant melanoma. Neoplastic cells with a diffuse and strong cytoplasmic immunolabelling for PDGFR α (purple staining) streptavidin–biotin–peroxidase method. Mayer's haematoxylin counterstaining. Scale bar: 50 μ M.

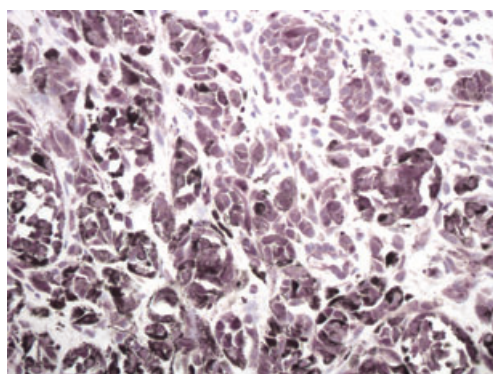


Figure 2. Malignant melanoma. Neoplastic cells with a diffuse and strong cytoplasmic immunolabelling for PDGFR β (purple staining) streptavidin–biotin–peroxidase method. Mayer's haematoxylin counterstaining. Scale bar: 50 μ M.

was statistically associated with both a shorter DFI (188 days versus 484 days – $P < 0.05$) and a shorter OS (median 224 days versus 484 days – $P < 0.05$) (Fig. 4). The number of the samples available did not allow the evaluation of the prognostic value of the single expression of PDGFR- α or - β . However, the expression of PDGFR- α was statistically associated with the expression of PDGFR- β (Chi square test, $P < 0.05$). Other evaluations comparing the IHC results with all the clinical or pathological data available did not show any statistical association. Besides, in this series of cases, no statistical differences in survival were found comparing patients of different clinical stage or those with complete and incomplete surgical excision. The Cox regression model for proportional hazard

assessment of PDGFR co-expression in interaction with high values of Ki67 was statistically significant ($P < 0.05$) and yielded odds ratio of 1.65 for the co-expression and 1.96 for the Ki67, respectively ($R^2 = 0.159$, log-rank test $P < 0.05$). However, the confidence intervals for the coefficients were quite wide owing to the limited sample size (0.77–3.57 for co-expression and 0.78–4.81 for Ki67).

Discussion

CMM is the most common oral malignancy in dogs and is generally locally aggressive and highly metastatic. Primary tumour *en bloc* resection and regional lymphadenectomy, with or without adjuvant radiotherapy, are the preferred methods of treatment and results in loco-regional control in up to 75% of CMMs. Disappointingly, the 1-year survival rate is less than 30%, even after adjuvant treatment; in particular, adjuvant chemotherapy does not result in a significant increase of the disease-free period.^{26–28} It may also be argued that the post-surgical outcome may be influenced by the clinical stage, but the authors of this paper did not reach any conclusion from the present data.²⁹ Immunotherapy against specific tumour associated antigens³⁰ has been employed in an adjuvant setting in an attempt to improve the life expectancy in case of CMM and results appear encouraging.^{31,32}

PDGFRs are physiologically expressed in a variety of cell types, such as fibroblasts, vascular smooth muscle cells and endothelial cells,¹⁰ suggesting a role also in the interaction between neoplastic cells and stromal compartment during tumour progression and invasion.²⁹ PDGFR- α and - β receptors are activated by specific soluble factors known as PDGF-A and -B that act as dimeric isoforms (PDGF-AA, -AB, and -BB) as well as the newly discovered protease activated isoforms PDGF-C and PDGF-D. PDGF-AA binds selectively to PDGFR- α , while PDGF-B chain isoforms bind and dimerize both PDGFR- α and PDGFR- β . In humans, several studies demonstrated the ability of PDGF ligands to interact with PDGFR- α and - β and induce homodimerization and/or heterodimerization of the receptors.^{33,34}

As shown previously, in our samples we found that the co-expression of both isoforms was higher

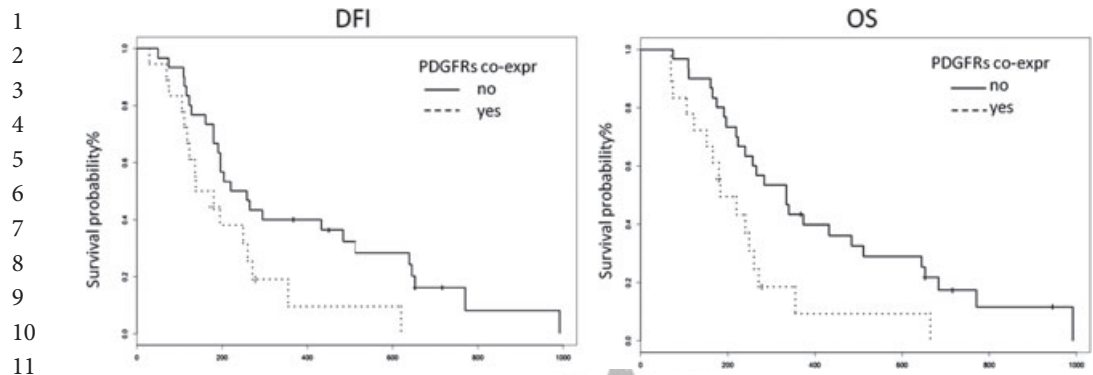


Figure 3. Kaplan–Meier curve of DFI (left box) in patients with melanoma co-expressing both PDGFR- α and - β (median 159 days) and not co-expressing PDGFR- α and - β (median 239 days – log-rank test: $P < 0.05$) and Kaplan–Meier curve of OS (right box) in patient co-expressing both PDGFR- α and - β (median 183 days) and not co-expressing PDGFR- α and - β (median 335 days – log-rank test: $P < 0.05$).

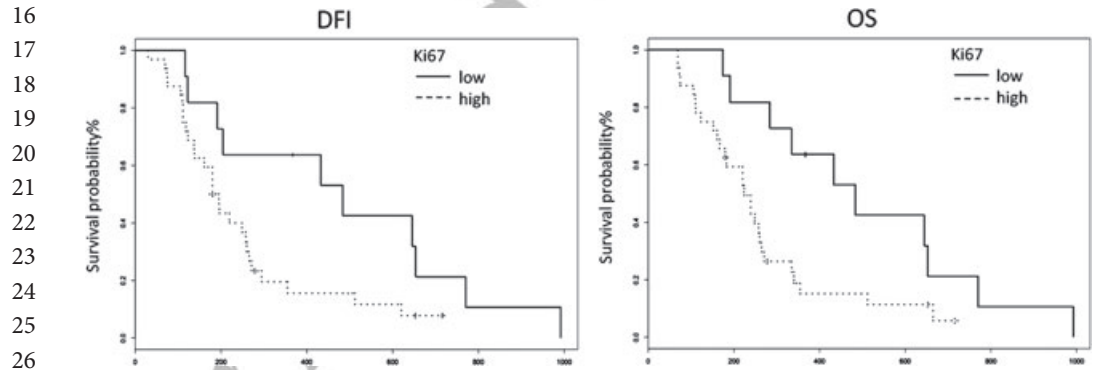


Figure 4. Kaplan–Meier curve of DFI (left box) in patient with Ki67 $> 19.5\%$ (median 188) and Ki67 $< 19.5\%$ (median 484 – log-rank test: $P > 0.05$) and Kaplan–Meier curve of OS (right box) in patient with melanoma positive (median 224 days) and negative for PDGFR- α (median 484 days – log-rank test: $P < 0.05$).

(37.5%) than the presence of PDGFR- α or - β alone (16.7 and 14.6%, respectively). This finding may suggest that these receptors can act independently by homodimerization as well as by heterodimerization.³⁵

A study on PDGFs and PDGFRs in human cutaneous melanomas³⁶ demonstrated that, at IHC, both the primary and metastatic melanoma exhibited significant expression of PDGF-AA, PDGF-BB and PDGF- α receptor when compared with normal skin, while no expression for PDGF- β receptor was recorded. These results have been recently confirmed by a study where PDGFR- α resulted overexpressed in a small population of human melanomas (4.6%) and an increased copy number was found.^{37–40} Contrary to human melanoma, in our sample PDGFR- β was expressed in 37.5% of samples, thus suggesting a different role. In the

present study, PDGFRs were detected not only in tumour tissue but also in the stromal compartment, suggesting a potential role in matrix remodelling and tumour invasion.¹²

In domestic animal tumours, the IHC expression of PDGFRs has been investigated in astrocytoma,¹⁹ lymphoma,¹⁷ osteosarcoma,¹⁶ anal sac adenocarcinoma,¹⁸ thyroid carcinoma⁴¹ and haemangiosarcoma,²⁰ highlighting the importance of these receptors also in the tumour biology of animals, as it occurs in humans.⁴² However, for none of these tumours a prognostic relevance has been demonstrated.

One important limitation of this study is its retrospective nature. Nevertheless, results show that the co-expression of PDGFR- α and PDGFR- β (37.5% of all CMMs of this series) is statistically associated to both DFI and OS ($P < 0.05$) and could therefore

be considered as a negative prognostic factor. This study also confirms the prognostic importance of Ki67,²⁴ whereas results regarding free versus infiltrated surgical margins, in the face of an *en bloc* surgery (CMM considered inoperable, i.e. with no chance to get clean margins at surgery, were not included here), and clinical stage failed to correlate with survival. Although this is an unexpected result, it should be considered that, as shown in another study dealing with a greater number of dogs with CMM, other factors such as the old age of the dogs and the size of the tumour may act as negative prognosticators.²⁷ It should also be noted that, in this study, no stage I CMMs was included and only stage II and III CMM were considered. Collectively, the data obtained from this study suggest that PDGFRs may play a role in the pathogenesis of CMM and the co-expression of both PDGFRs- α and - β should be taken in account as a negative prognostic marker. Further prospective studies on a greater number of cases are warranted to confirm this finding.

Acknowledgements

Authors wish to thank Alessandra Sereno for technical support, Dr. Luca Rotolo for statistical support and the Reference Centre of Comparative Pathology 'Bruno Maria Zaini' of the Department of Veterinary Science of Grugliasco.

Conflict of interest

None of the authors have financial or personal relationships that could inappropriately influence or bias the content of the paper.

References

1. Bergman PJ. Canine oral melanoma. *Clinical Techniques in Small Animal Practice* 2007; **22**: 55–60.
2. Ramos-Vara JA, Beissenherz ME, Miller MA, Johnson GC, Pace LW, Fard A, *et al.* Retrospective study of 338 canine oral melanomas with clinical, histologic, and immunohistochemical review of 129 cases. *Veterinary Pathology* 2000; **37**: 597–608.
3. Smith SH, Goldschmidt MH and McManus PM. A comparative review of melanocytic neoplasms. *Veterinary Pathology* 2002; **39**: 651–678.
4. Smedley RC, Spangler WL, Esplin DG, Kitchell BE, Bergman PJ, Ho HY, *et al.* Prognostic markers for canine melanocytic neoplasms: a comparative review of the literature and goals for future investigation. *Veterinary Pathology* 2011; **48**: 54–72.
5. Gillard M, Cadieu E, De Brito C, Abadie J, Vergier B, Devauchelle P, *et al.* Naturally occurring melanomas in dogs as models for non-UV pathways of human melanomas. *Pigment Cell & Melanoma Research* 2014; **27**.
6. Bauer J, Curtin JA, Pinkel D and Bastian BC. Congenital melanocytic nevi frequently harbor NRAS mutations but no BRAF mutations. *Journal of Investigative Dermatology* 2007; **127**: 179–182.
7. Liu F, He K, Yang X, Xu N, Liang Z, Xu M, *et al.* α 1A-adrenergic receptor induces activation of extracellular signal-regulated kinase 1/2 through endocytic pathway. *PLoS ONE* 2011; **6**: e21520.
8. Demoulin J-B and Essaghir A. PDGF receptor signaling networks in normal and cancer cells. *Cytokine & Growth Factor Reviews* 2014; **25**: 273–283.
9. Alvarez RH, Kantarjian HM and Cortes JE. Biology of platelet-derived growth factor and its involvement in disease. *Mayo Clinic Proceedings* 2006; **81**: 1241–1257.
10. Toffalini F and Demoulin J-B. New insights into the mechanisms of hematopoietic cell transformation by activated receptor tyrosine kinases. *Blood* 2010; **116**: 2429–2437.
11. Ostman A and Heldin C-H. PDGF receptors as targets in tumor treatment. *Advances in Cancer Research* 2007; **97**: 247–274.
12. Paulsson J, Ehnman M and Ostman A. PDGF receptors in tumor biology: prognostic and predictive potential. *Future Oncology* 2014; **10**: 1695–1708.
13. Peng Y, Guo J-J, Liu Y-M and Wu X-L. MicroRNA-34A inhibits the growth, invasion and metastasis of gastric cancer by targeting PDGFR and MET expression. *Bioscience Reports* 2014; **34**: 247–256.
14. London CA. Tyrosine kinase inhibitors in veterinary medicine. *Topics in Companion Animal Medicine* 2009; **24**: 106–112.
15. Lachowicz JL, Post GS and Brodsky E. A phase I clinical trial evaluating imatinib mesylate (Gleevec) in tumor-bearing cats. *Journal of Veterinary Internal Medicine* 2005; **19**: 860–864.
16. Maniscalco L, Iussich S, Morello E, Martano M, Biolatti B, Riondato F, *et al.* PDGFs and PDGFRs in canine osteosarcoma: new targets for innovative therapeutic strategies in comparative oncology. *Veterinary Journal* 2013; **195**: 41–47.

17. Aricò A, Guadagnin E, Ferraresso S, Gelain ME, Iussich S, Rütgen BC, *et al.* Platelet-derived growth factors and receptors in Canine Lymphoma. *Journal of Comparative Pathology* 2014; **151**: 322–328.
18. Brown RJ, Newman SJ, Durtschi DC and Leblanc AK. Expression of PDGFR- β and Kit in canine anal sac apocrine gland adenocarcinoma using tissue immunohistochemistry. *Veterinary and Comparative Oncology* 2012; **10**: 74–79.
19. Higgins RJ, Dickinson PJ, LeCouteur RA, Bollen AW, Wang H, Wang H, *et al.* Spontaneous canine gliomas: overexpression of EGFR, PDGFR alpha and IGFBP2 demonstrated by tissue microarray immunophenotyping. *Journal of Neuro-Oncology* 2010; **98**: 49–55.
20. Asa SA, Murai A, Murakami M, Hoshino Y, Mori T, Maruo K, *et al.* Expression of platelet-derived growth factor and its receptors in spontaneous canine hemangiosarcoma and cutaneous hemangioma. *Histology and Histopathology* 2012; **27**: 601–607.
21. Williams LE and Packer RA. Association between lymph node size and metastasis in dogs with oral malignant melanoma: 100 cases (1987–2001). *Journal of the American Veterinary Medical Association* 2003; **222**: 1234–1236.
22. Owen LN. *TNM Classification of Tumours in Domestic Animals*. Geneve, 1980.
23. Ottnot JM, Smedley RC, Walshaw R, Hauptman JG, Kiupel M and Obradovich JE. A retrospective analysis of the efficacy of Oncept vaccine for the adjunct treatment of canine oral malignant melanoma. *Veterinary and Comparative Oncology* 2013; **11**: 219–229.
24. Bergin IL, Smedley RC, Esplin DG, Spangler WL and Kiupel M. Prognostic evaluation of ki67 threshold value in canine oral melanoma. *Veterinary Pathology* 2011; **48**: 41–53.
25. Donnem T, Al-Saad S, Al-Shibli K, Andersen S, Busund L-T and Bremnes RM. Prognostic impact of platelet-derived growth factors in non-small cell lung cancer tumor and stromal cells. *Journal of Thoracic Oncology* 2008; **3**: 963–970.
26. Dank G, Rassnick KM, Sokolovsky Y, Garrett LD, Post GS, Kitchell BE, *et al.* Use of adjuvant carboplatin for treatment of dogs with oral malignant melanoma following surgical excision. *Veterinary and Comparative Oncology* 2014; **12**: 78–84.
27. Boston SE, Lu X, Culp WTN, Montinaro V, Romanelli G, Dudley RM, *et al.* Efficacy of systemic adjuvant therapies administered to dogs after excision of oral malignant melanomas: 151 cases (2001–2012). *Journal of the American Veterinary Medical Association* 2014; **245**: 401–407.
28. Brockley LK, Cooper MA and Bennett PF. Malignant melanoma in 63 dogs (2001–2011): the effect of carboplatin chemotherapy on survival. *New Zealand Veterinary Journal* 2013; **61**: 25–31.
29. Tuohy JL, Selmic LE, Worley DR, Ehrhart NP and Withrow SJ. Outcome following curative-intent surgery for oral melanoma in dogs: 70 cases (1998–2011). *Journal of the American Veterinary Medical Association* 2014; **245**: 1266–1273.
30. Mayayo SL, Prestigio S, Maniscalco L, La Rosa G, Arico A, De Maria R, *et al.* Chondroitin sulfate proteoglycan-4: a biomarker and a potential immunotherapeutic target for canine malignant melanoma. *Veterinary Journal (London, England : 1997)* 2011; **190**: e26–e30.
31. Bergman PJ, McKnight J, Novosad A, Charney S, Farrelly J, Craft D, *et al.* Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. *Clinical Cancer Research* 2003; **9**: 1284–1290.
32. Riccardo F, Iussich S, Maniscalco L, Mayayo SL, La Rosa G, Arigoni M, *et al.* CSPG4-specific immunity and survival prolongation in dogs with oral malignant melanoma immunized with human CSPG4 DNA. *Clinical Cancer Research* 2014; **20**: 3753–3762.
33. LaRochelle WJ, Jeffers M, McDonald WF, Chillakuru RA, Giese NA, Lokker NA, *et al.* PDGFD, a new protease-activated growth factor. *Nature Cell Biology* 2001; **3**: 517–521.
34. Heidaran MA, Pierce JH, Yu JC, Lombardi D, Artrip JE, Fleming TP, *et al.* Role of alpha-beta-receptor heterodimer formation in beta-platelet-derived growth-factor (PDGF) receptor activation by PDGF-AB. *Journal of Biological Chemistry* 1991; **266**: 20232–20237.
35. Ostman A. PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. *Cytokine & Growth Factor Reviews* 2004; **15**: 275–286.
36. Barnhill RL, Xiao M, Graves D and Antoniades HN. Expression of platelet-derived growth factor (PDGF)-A, PDGF-B and the PDGF-alpha receptor, but not the PDGF-beta receptor, in human malignant melanoma in vivo. *British Journal of Dermatology* 1996; **135**: 898–904.
37. Terada T. Low incidence of KIT gene mutations and no PDGFRA gene mutations in primary cutaneous melanoma: an immunohistochemical and molecular genetic study of Japanese cases. *International Journal of Clinical Oncology* 2010; **15**: 453–456.

38. Dai J, Kong Y, Si L, Chi Z, Cui C, Sheng X, *et al.* Large-scale analysis of PDGFRA mutations in melanomas and evaluation of their sensitivity to tyrosine kinase inhibitors imatinib and crenolanib. *Clinical Cancer Research* 2013; **19**: 6935–6942.
39. McGary EC, Onn A, Mills L, Heimberger A, Eton O, Thomas GW, *et al.* Imatinib mesylate inhibits platelet-derived growth factor receptor phosphorylation of melanoma cells but does not affect tumorigenicity in vivo. *Journal of Investigative Dermatology* 2004; **122**: 400–405.
40. Wallander ML, Layfield LJ, Emerson LL, Mamalis N, Davis D, Tripp SR, *et al.* KIT mutations in ocular melanoma: frequency and anatomic distribution. *Modern Pathology* 2011; **24**: 1031–1035.
41. Urie BK, Russell DS, Kisseberth WC and London CA. Evaluation of expression and function of vascular endothelial growth factor receptor 2, platelet derived growth factor receptors-alpha and -beta, KIT, and RET in canine apocrine gland anal sac adenocarcinoma and thyroid carcinoma. *BMC Veterinary Research* 2012; **8**: 67.
42. Kikuchi A and Monga SP. PDGFRalpha in liver pathophysiology: emerging roles in development, regeneration, fibrosis, and cancer. *Gene Expression* 2015; **16**: 109–127.

QUERIES TO BE ANSWERED BY AUTHOR

IMPORTANT NOTE: Please mark your corrections and answers to these queries directly onto the proof at the relevant place. DO NOT mark your corrections on this query sheet.

Queries from the Copyeditor:

- AQ1.** Please confirm that given names (red) and surnames/family names (green) have been identified correctly
- AQ2.** Kindly check whether the insert is correct.
- AQ3.** Please give address information for Santa Cruz Biotechnology and Dako: city, state (if USA), and country.
- AQ4.** Please give address information for Vector Laboratories: city, state (if USA), and country.
- AQ5.** Figure 4 was not cited in the text. An attempt has been made to insert the figure into a relevant point in the text –please check that this is OK. If not, please provide clear guidance on where it should be cited in the text.
- AQ6.** These figures (3, 4) are poor quality. Kindly resupply.
- AQ7.** Please provide Page information for Ref. [5].
- AQ8.** Please provide Publisher name for Ref. [22].
-